THE CHEMICAL COMPOSITION OF CHLORELLA; EFFECT OF ENVIRONMENTAL CONDITIONS

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Introduction

The purpose of the investigations here described was to determine to what extent the chemical composition of a plant can be altered by growing it under different environmental conditions. The three general processes which determine the organic composition of a plant are photosynthesis, respiration, including aerobic and anaerobic catabolism, and biosynthesis. In the present state of knowledge it is difficult to discern to what extent these three processes are interrelated. For the immediate purposes of this investigation it is, in fact, not essential to attempt to determine which of these processes may be responsible for any observed changes in composition. If decided changes in composition are observable as results of altered environmental conditions, these effects can then be further analyzed and can perhaps ultimately be ascribed to one or another of the major processes. The immediate problem was to discover which environmental conditions would, when altered, affect the organic composition of the plant, and to determine the nature and magnitude of the effect. The objective was to find environmental conditions which would materially modify the relative proportions of the three major plant constituents, carbohydrates, proteins and lipids.

For such an undertaking, very little information was available as a basis for a working hypothesis to aid in the choice of environmental conditions. It was obvious from the outset, therefore, that a large number of experiments would have to be made.

In an investigation requiring many analyses, the determination of the organic composition of the plants in terms of definite chemical compounds, or even of groups of compounds, would be very laborious and time consuming. Regarding all of the organic carbon of the plant as arising from the reduction of carbon dioxide, we were more concerned with the energy level of this total organic matter than with the isolation of particular constituents. The determination of the heat of combustion of the plant material would, in a measure, satisfy this requirement; but such data would have a limited usefulness. Recourse was taken to a very simple means of determining the "degree of reduction" of the entire organic material of the plant from its elementary chemical composition. This general concept, which has been used but little in connection with problems of this nature,

also permits calculation of the proportions of the major plant constituents and will be discussed in the first section of this paper.

The choice of the plant material for such an investigation is doubtless of great importance. Higher plants are probably not very flexible in respect to their organic composition. They have certain set requirements already imposed by their elaborate structural elements, which constitute a large portion of their total substance. It is conceivable that lower organisms have not such set requirements, and that their degree of flexibility in regard to chemical composition may be greater. The unicellular green algae, for example, grow rapidly and permit relative simplicity in conducting controlled culture experiments. For this reason, and because its extensive use in the study of photosynthesis afforded considerable information concerning its behavior, *Chlorella pyrenoidosa* was used in the present investigations. It was found to be very suitable for such experiments because it grows well under a wide variety of conditions.

It is hoped that the results here described may have some significance in the interpretation of data obtained in photosynthesis experiments. In conjunction with the determinations of the chemical composition of Chlorella cells grown under different conditions, it was originally planned to carry out determinations of the rate of photosynthesis and of respiratory and photosynthetic quotients of cells from the same cultures. Unfortunately, conditions imposed by the war forced postponement of this part of the program, and since then it has not been possible to undertake it.

The Degree of Reduction of Carbon and the R-Value

We are here concerned with the unique ability of the green plant to use carbon dioxide and water as its principal raw materials and to elaborate from the carbon and hydrogen contained in them a host of organic compounds, often molecules of great complexity. The raw materials have a high oxygen content, 72.71% in carbon dioxide and 88.81% in water. The constituents of the plant have a much lower percentage of oxygen. For example, analyses of entire Chlorella cells show a range of oxygen content from 34% to as low as 18%.

It is apparent that the plant has, in the synthesis of its components, eliminated a large part of the oxygen content of the carbon dioxide and water. From a chemical viewpoint this is a process of reduction. An input of energy is required to make the process go, and the reduced compounds which are formed represent a storage of energy. The level of reduction to which the carbon has been carried represents the amount of energy stored. A numerical expression of the level or degree of reduction of carbon and of the energy content of carbon compounds can be derived through the application of simple chemical principles.

The degree of reduction of the carbon in an organic compound is related to the percentage of carbon, hydrogen, and oxygen. Since oxidation

and reduction are the reverse of one another, the most oxidized carbon compound, carbon dioxide, may equally well be spoken of as the least reduced form of carbon. Hence, carbon dioxide has a degree of reduction of zero. The most reduced carbon compound, which represents the highest degree of reduction of carbon, is methane. The degree of reduction of carbon in all other organic compounds will, then, be intermediate between that of carbon dioxide and that of methane.

The degree of reduction of an organic compound can be measured by oxidation. Complete oxidation converts all of the carbon to carbon dioxide and all of the hydrogen to water. The energy released as heat in the combustion is equal to the energy stored by the reduction. The amount of oxygen required to accomplish complete oxidation of an organic compound is a measure of both the degree of reduction and of the energy stored in the compound.

The ratio of oxygen to carbon and to hydrogen for their complete oxidation to carbon dioxide and water is expressed by:

$$\frac{O_2}{C} = \frac{32}{12.01} = 2.664 \text{ and } \frac{O}{H_2} = \frac{16}{2.016} = 7.936$$

The degree of reduction can be expressed by: $(\% C \times 2.664) + (\% H \times 7.936)$. Starting with the most highly reduced carbon compound, methane, which contains 74.87% carbon and 25.13% hydrogen, we calculate its degree of reduction as:

$$(74.87 \times 2.664) + (25.13 \times 7.936) = 398.9$$

In words, methane combines with 398.9% its own weight of oxygen during complete combustion to carbon dioxide and water.

In the case of a compound containing oxygen, less oxygen will be required for complete oxidation for two reasons. Firstly, because of the presence of the oxygen in the compound, the percentages of carbon and hydrogen are diminished. In the second place, because the compound contains oxygen, the carbon may be considered to be already partially oxidized. Therefore, to find the degree of reduction of a compound containing oxygen, one must calculate how much more oxygen is required to complete the oxidation of the carbon and hydrogen to carbon dioxide and water. This is done by calculating, as before, the total oxygen required, then subtracting the percentage of oxygen already present. Take for an example methanol, which has 37.48% carbon, 12.58% hydrogen and 49.94% oxygen. Its degree of reduction is expressed by:

$$(37.48 \times 2.664) + (12.58 \times 7.936) - 49.94 = 149.8.$$

The remaining element to be considered in the calculation of the degree of reduction is nitrogen. This is regarded as inert. The nitrogen content of the compounds with which we shall deal is predominantly in the form of amino groups. In the complete oxidation of this type of compound,

the nitrogen appears in the elementary form among the products of combustion. Therefore, its effect upon the degree of reduction of the compound is due merely to the fact that its presence diminishes the percentage of carbon and hydrogen in the compound. For example, consider two compounds which differ in empirical formula only by two nitrogen atoms:

Propanol	C_3H_8O	59.96% C,	13.42% H,	26.62% O
Ethyl urea	$C_3H_8ON_2$	48.62% C,	10.88% H,	21.59% O

The degree of reduction of these compounds, calculated as shown for methanol, is: propanol, 239.6 and ethyl urea, 194.3.

THE R-VALUE. The degree of reduction as calculated for the foregoing compounds is an inconvenient number for comparison of values. We have, therefore, used a derived scale to express the R-value. Since carbon dioxide has a reduction level of zero, it is zero on the R-value scale also. Because methane is the most highly reduced carbon compound, it is assigned an R-value of 100. The degree of reduction of any other organic compound can be expressed as its *percentage* of the degree of reduction of methane. This percentage value is from now on referred to as the R-value. The general formula for calculation of R-value is expressed by:

$$\frac{[(\%\ C \times 2.664) + (\%\ H \times 7.936) - \%\ O] \times 100}{398.9} = \text{R-value}$$

So far we have considered specific chemical compounds. The concept of R-value is equally applicable to mixtures of organic compounds, even when the chemical structure of these is unknown. Therein lies its application in the present investigation. We are interested in the total organic material produced during the life of a Chlorella culture; the level of reduction which the carbon of the materials comprising the entire plant has reached; how much energy has been stored in the over-all process.

The four elements, carbon, hydrogen, nitrogen and oxygen add up to nearly 100% in the analysis of the organic material making up a whole plant. The first three are determined by combustion analysis of the plant and are expressed upon an ash-free basis. The difference between 100% and the sum of the percentages of carbon, hydrogen and nitrogen is reported as per cent. oxygen. The R-value of the material is then calculated by the general formula given above.

It is realized that sulfur and phosphorus are also constituents of plant material, that they too affect the oxygen requirement for complete oxidation of the material and so affect the R-value. However, these elements are present in such small percentages that their effect upon the R-value of the plant material is considered negligible, within the range of the over-all experimental errors. If sulphur and phosphorus were determined, their effect would be to increase the reported R-values by a very slight amount. Even one per cent. sulfur, an enormous amount in terms of an entire plant, would raise the calculated R-value by only 0.4 unit. The effect of one per

cent. phosphorus would be still less, since it is probably present as phosphate, already oxidized, and would in fact, largely appear in the ash.

Although its calculation is based upon the degree of reduction of carbon in organic compounds, the R-value is more directly an expression of the energy content of organic materials. It is directly proportional to the heat of combustion *per gram* (THORNTON, **18**). As an energy unit it has greater value in measuring the over-all effect of photosynthetic and metabolic activity than other units which might have been used. A scale of reduction based upon molecular quantities, for instance, is not applicable to material

TABLE I

CHEMICAL COMPOSITION AND R-VALUE OF SOME BIOLOGICALLY IMPORTANT COMPOUNDS

COMPOUND	FORMULA	% C	% H	% N	% O	R-VALUE
Methane	CH_4	74.87	25.13			100.00
Isoprene	C_5H_8	88.16	11.84			82.43
Carotene	$C_{40}H_{56}$	89.48	10.52			80.68
Triolein	$C_{57}H_{104}O_6$	77.32	11.84		10.84	72.48
Chlorophyll a	$C_{55}H_{72}O_5N_4Mg$	73.93	8.12	6.27	8.95	63.28
Leucine	$C_6H_{13}O_2N$	54.93	9.99	10.70	24.40	50.45
Asparagine	$C_4H_8O_3N_2$	36.36	6.10	21.21	36.33	27.31
Ethanol	C_2H_6O	52.14	13.13		34.73	52.23
Glycerol	$C_3H_8O_3$	39.12	8.76		52.12	30.48
Starch	$(C_6H_{10}O_5)_n$	44.44	6.22		49.34	29.70
Glucose	$C_6H_{12}O_6$	40.00	6.71		53.29	26.69
Pyruvic acid	$C_3H_4O_3$	40.91	4.58		54.51	22.75
Malic acid	$C_4H_6O_5$	35.83	4.51		59.66	17.94
Oxalic acid	$C_2H_2O_4$	26.68	2.24		71.08	4.46
Carbon dioxide	CO_2	27.29			72.71	0

which is a mixture of organic substances, some of great complexity and some of unknown structure. Moreover, the elementary analyses from which R-values are calculated give additional chemical information about the materials investigated. The chemical analyses and R-values of some organic compounds of biological importance are shown in table I.

Methods

CULTURE OF CHLORELLA PYRENOIDOSA

All of the cultures herein described were inoculated with descendants from a single slant of *Chlorella pyrenoidosa* (Emerson's strain). This stock was maintained during the course of the experiments by transfer onto fresh agar slants. From a slant the alga was put into liquid culture in test tubes. The test tube cultures were used to inoculate "stock cultures". Stock cultures were grown in 500 ml. flasks fitted with rubber stoppers carrying glass tubes for aeration and transfer. Each experimental culture was inoculated by aseptic transfer of 15 ml. of a stock culture. When it was desired to start a number of experimental cultures with identical inocula, one stock culture served to inoculate as many as 10 larger cultures.

The experimental cultures were grown in Fernbach flasks closed with rubber stoppers. Each stopper carried three glass tubes: a short wide tube with cotton plug, serving as a gas outlet; a short, bent tube for introduction of inoculum; a gas inlet tube reaching to the bottom of the flasks. The tube for inoculation was, of course, plugged with cotton and a cotton-filled filter was attached to the gas inlet tube. The flask containing 2000 ml. of mineral nutrient solution was fitted with stopper, tubes and filter. Cotton batting was wrapped to a thickness of about an inch around the neck of the flask, stopper and tubes and was tied in place. The entire assembly was then sterilized by steam for 20 minutes at 15 pounds pressure.

After the cultures were inoculated, they were allowed to grow for the desired length of time under a given set of environmental conditions. The apparatus used to control these conditions was quite simple. A wooden framework, open on the sides and with a circular opening in the top, formed the support. An incandescent lamp was centrally placed below the top opening. A reflector below and around the lamp directed the light upward. The culture flask rested upon glass rods in a glass dish, which in turn was supported in the circular opening in the top of the framework. The distance from the lamp bulb to the bottom of the culture flask was 20 cm.

The light intensity incident upon the culture was dependent upon the rating of the frosted Mazda lamps. Lamps ranging from 25-watt to 500-watt capacity were used in various experiments. Unless otherwise noted, illumination of the cultures was continuous throughout their period of growth. Water was circulated through the glass dish in which the culture flask stood. The rate of flow of the water served to maintain the culture at any desired temperature.

A gas mixture of predetermined composition was continuously bubbled through the cultures. The agitation caused by bubbling largely prevented settling of the Chlorella cells. In addition, the cultures were shaken by hand several times daily. The gas mixtures were either purchased in cylinders or, in the case of carbon dioxide in air, prepared with use of a compressor unit. The other factor subjected to experimental variation was the composition of the nutrient solution.

The number of days allotted for the growth of each culture varied with the experimental objectives. At the end of the time selected, each culture was examined microscopically for contamination. Very few of the cultures became contaminated. If any organism other than Chlorella was found, the culture was discarded. All analytical data presented in this paper are, therefore, based upon *pure* cultures of Chlorella, not upon merely unialgal cultures.

The cultures found to be pure were quickly cooled to about 2° C. The cells were separated from the medium in a centrifuge. The sedimented cells were resuspended in a little distilled water to rinse off entrained nu-

trient solution, following which they were again centrifuged sharply. The fresh weight of the cells was recorded at this point. The cells were put immediately into a vacuum desiccator to dry at room temperature over calcium chloride. After 24 hours the desiccant was changed and the drying in vacuum was continued until the cells reached constant weight, usually 48 to 72 hours altogether.

Analytical Methods

Since the objective of this work was to discover what changes in chemical composition of an alga could be induced by changes in the environmental conditions under which it grows, it was important to account for all of the organic matter produced. The completely cell-free solutions from several cultures were analyzed for organic matter. The total organic carbon found in the cell-free culture solutions varied from 2.4 to 3.9% of the total carbon content of the cells themselves. From this it is clear that analysis of the cells represents from 96 to 98% of the total organic matter produced by a Chlorella culture. The small amount of organic matter found in solution in the culture medium was not examined further.

The dry cell mass was pulverized in an agate mortar until all of it passed through a 60-mesh screen. A sample was then taken for carbon-hydrogen analysis. Another sample was then ground as fine as possible in the agate mortar and was used for the nitrogen determination.

Samples of Chlorella cells for combustion were handled with the usual precautions applying to hygroscopic materials. Each sample was dried in vacuum over phosphorus pentoxide. Sample and boat were protected in a "piggie" during weighing and transfer.

Nitrogen analyses were made by the micro-Dumas method as described by Pregl.

Carbon and hydrogen were determined by the macro method. The combustion tube filling described by Fisher (5) was used. The samples were burned in platinum boats. The unburned residue from the carbon-hydrogen analysis was reported as per cent. ash.

Complete combustion of material such as Chlorella in the carbon-hydrogen analysis offers some difficulty. Being a mixture of carbohydrate, protein and lipid materials, the sample exhibits a combination of the annoying features of each during combustion. Samples having different compositions behave differently while burning. In general, it is advisable to proceed slowly and to use as low a temperature in the boat-heating unit of the furnace as will insure complete combustion. At first the sample decomposes and tarry substances distill from the boat and condense on the tube. By heating slowly, these deposits can be burned clean. After the decomposition products have left the combustion boat, the carbonaceous residue will ignite. Usually it burns clean, leaving a light colored, fluffy ash. The character of the ash was found to vary with the mineral nutrient

medium in which the sample of Chlorella had grown. High nitrate media yielded Chlorella, the ash from which gave unusual difficulty in the carbon-hydrogen analysis. The melting point of this ash was so low that frequently the ash would fuse during the ignition of the sample. Sometimes carbon was left in this fused ash, and resisted the usual devices for combustion of the last traces. In such cases, the mixture of ash and unburned carbon was weighed, washed with dilute hydrochloric acid, and the carbon determined separately. The weight of this residual carbon was subtracted from the weight of ash plus carbon to get the weight of the ash, and was added to the main carbon determination to obtain the correct carbon content of the sample. Only a few samples required this treatment.

Calculation of the R-value

From the percentages of carbon, hydrogen, nitrogen and ash, determined as described, the R-value of the organic matter produced by each of the Chlorella cultures was calculated. The R-value applies only to the organic constituents of the entire cell mass, hence the percentages of carbon, hydrogen, and nitrogen were calculated to an ash-free basis. The oxygen content of the organic matter was taken as 100% less the sum of the percentages of ash-free carbon, hydrogen and nitrogen. Phosphorus and sulfur were disregarded, as explained in the section on R-value. By use of the general formula given there and the percentages of carbon, hydrogen and oxygen expressed on the ash-free basis, the R-value of the Chlorella was computed.

Composition and R-Value

Dry Weight

Because we are concerned with the quantity and composition of the organic matter produced by Chlorella cultures, all cell yields are expressed as grams dry weight per two liter culture. This is an important point for questions under consideration. Frequently the larger of two yields in fresh weight is the smaller in dry weight, or vice versa. In series of cultures which differed only in the length of time which was allowed for growth, it was sometimes found that the yield in grams fresh weight actually decreased, whereas the yield in grams dry weight from the same cultures showed a steady increase with age. The cell count is also an unreliable index of the weight of organic matter produced, because Chlorella cells vary greatly in size at different times in the life of the culture under some environmental conditions.

The per cent. dry weight of the Chlorella cells obtained from a culture does not enter into the determination of the composition or R-value. This percentage is not without interest, however. Under widely different cultural conditions, the per cent. dry weight of Chlorella was observed to vary from 11 to 42% of the fresh weight. The usual range of this value in

most of the cultures grown was 20 to 33%. The composition and concentration of the mineral nutrients appear to have an effect upon the per cent. dry weight of Chlorella. Under a given set of conditions, the per cent. dry weight tends to increase with the yield of cells and the age of the culture. Cells of high R-value tend to have a high per cent. dry weight. This is probably because they are grown for a long time and because they contain a greater proportion of non-hydrophylic constituents.

Ash

No analyses were made of the composition of the ash of Chlorella. Its percentage was determined in order to calculate the percentages of carbon, hydrogen and nitrogen to an ash-free basis in the computation of R-value. The percentage of inorganic material entering into the composition of Chlorella cells is largely influenced by the proportions and concentrations of the mineral salts in the nutrient medium. To a lesser extent the per cent. ash depends upon the yield of cells produced. The ash content decreases as more organic matter is produced. The range observed in ash content was from 1.5 to 20%. The majority of the cultures produced Chlorella having an ash content of 2 to 10%. The cells of high R-value usually had less than 5% ash.

Carbon and Hydrogen

As would be expected from the way in which R-value is calculated, the carbon and hydrogen content of Chlorella cells bear a linear relationship to the R-value. The small variations from linearity are readily explained in relation to the varying nitrogen content of the samples. The per cent. carbon, on an ash-free basis, varied from 49.5% at the lowest R-value to 70.2% in the sample of highest R-value. The corresponding figures for per cent. hydrogen, ash-free, were 6.78% and 10.53%.

The atomic ratio of hydrogen to carbon was calculated for a number of Chlorella samples. This ratio proved to have little value for our purpose because of the considerable variation in nitrogen content between samples having nearly the same carbon and hydrogen content. The hydrogen to carbon ratio varied only from about 1.6 to 1.8, while the R-value changed from 42 to 55 for the samples compared by both methods.

Nitrogen and Oxygen

The relation of nitrogen content of Chlorella to R-value will be discussed in another section of this paper. The observed range of nitrogen content was from 1.17 to 14.11%. In general, the per cent. nitrogen decreases with increase in R-value, but the relation is not a simple one.

The percentage of oxygen in Chlorella also decreases as the R-value rises. Here again the relation is not linear because of the fluctuation of nitrogen content. If, however, the sum of per cent. nitrogen and per cent.

oxygen is plotted against the R-value, the points lie very close to a straight line. This sum decreases from 43.5 to 19.5% as the R-value increases from the lowest to the highest observed.

R-Value of Chlorella

In these experiments the R-values varied from 38 to 63. The change in R-value during the growth of a culture is continuous rather than stepwise. Three hundred and twenty cultures were analyzed. The distribution of observed R-values between the lowest and the highest is remarkably uniform.

In table II are shown the relation between R-value and elementary

TABLE II

Variation in composition and R-value of chlorella

R-value	Calculated on ash-free basis:				
	% Ash	% C	% H	% N	% O
37.92	3.45	49.51	6.78	9.31	34.40
40.08	3.78	51.00	6.90	11.29	30.81
42.41	20.21	51.65	7.37	14.11	26.87
43.99	5.81	54.59	7.28	10.38	27.75
43.99	8.56	54.79	7.93	5.28	32.00
45.92	7.88	54.58	8.11	10.75	26.56
45.92	2.28	56.01	7.84	7.86	28.29
50.18	2.87	59.73	8.56	4.80	26.91
54.37	1.36	63.77	8.97	3.08	24.18
57.05	5.32	65.35	9.68	1.62	23.35
60.29	4.57	68.18	10.01	1.28	20.53
61.87	3.44	69.19	10.33	1.17	19.31
63.33	3.46	70.17	10.53	1.43	17.87

composition, the range covered by these values, and the differences in composition which may be found in samples of the same R-value.

R-Value of Other Plant Material

The R-values of a number of other plants were determined. Many of these plants had grown under natural conditions. The R-values of the algae analyzed were as follows: *Amphipleura rutilans* 33.76; *Navicula torquatum* 36.22; *Nitzschia closterium* 40.05; *Anabaenopsis* sp. 37.88; *Peridinium cinctum* 41.93; *Ulva* sp. 30.53: *Chlamydomonas* sp. 35.27; *Stichococcus bacillaris* 43.89; *Gigartina Agardhii* 29.85; of the following the blades only were analyzed: *Macrocystis pyrifera* 34.24; *Egregia Menziesii* 36.27.

The dried leaves of several common plants were also analyzed and showed the following R-values: *Zea Mays* 31.88; *Medicago sativa* 38.00; *Helianthus annuus* 38.37; *Linum usitatissimum* 40.08.

That the R-value of leaves may vary with the composition of the leaves

is illustrated by the following. Nasturtium leaves with a starch content of 2% had an R-value of 38.45, and those with a starch content of 20% had an R-value of 34.44. Similarly, tobacco leaves with starch content of 2.5% and 44% had R-values of 38.77 and 34.26 respectively. Obviously, the accumulation of a substance having a low R-value such as starch, results in a decrease of the R-value of the whole leaf.

It is of interest that in the complicated processes comprising the conversion of vegetable material to fuels of higher heating values, there occurs a notable decrease in the oxygen content. This change is accompanied by a marked increase in the carbon content, in the R-value, and hence also in the calorific value, of the material which has undergone this type of conversion. The elementary composition and the R-value of some of these products are given in table III.

TABLE III

COMPOSITION AND R-VALUES OF SOME FUELS

MATERIAL	CALCULATED ON AN ASH-FREE BASIS:				
	% C	% H	% N	% O	R-VALUE
Wood, Basswood	49.05	5.88	0.62	44.45	33.32
" Sugar maple	49.52	5.99	0.69	43.80	34.00
Peat*	55.54	6.28	1.72	36.56	38.18
Lignite*	72.95	5.24	1.31	20.50	53.56
Bituminous Coal*	84.24	5.55	1.52	8.69	64.74
Anthracite Coal*	93.50	2.81	0.97	2.72	67.09
Petroleum	87.94	11.21	0.85		80.82

* Analyses taken from F. W. Clarke, The Data of Geochemistry, U. S. Geological Survey, Bulletin 770, p. 773, 1924.

CONSTITUENTS OF CHLORELLA

The elementary composition and R-value of Chlorella are obtainable with relatively little effort compared to the tedious analysis of plant material for carbohydrate, protein and fat content which are of great interest and importance from a physiological viewpoint. Fortunately it is easy to calculate the approximate lipid, protein and carbohydrate content from the elementary analysis and R-value.

We may take 28 as the R-value of the carbohydrate fraction, a value between that of hexose and of polysaccharide. From elementary analyses of proteins which are available in the literature, an R-value of 42 is obtained. Calculated from published fat analyses, the R-value of plant fats is about 67.5. This value is taken to apply to the total lipid fraction of Chlorella. Per cent. nitrogen multiplied by 6.25 is used as the per cent. protein. With this value as one constant and the R-value of the entire sample as another, algebraic solution of simultaneous equations gives the calculated percentage of carbohydrate and of lipid. Using P for protein,

C for carbohydrate and L for lipid the equations are:

$$(\%\ P \times 42) + (\%\ C \times 28) + (\%\ L \times 67.5) = \text{R-value} \times 100\%$$
$$\%\ P + \%\ C + \%\ L = 100$$

For example take Chlorella with 4.53% nitrogen and R-value 50:

$$4.53 \times 6.25 = 28.3\%\ P.\quad \text{Then, } C + L = 71.7\%$$

and, $(\%\ C \times 28) + (\%\ L \times 67.5) = (50 \times 100\%) - (28.3 \times 42)$. Solving; carbohydrate = 26.2% and lipid = 45.5%.

Calculations made by this method indicate remarkable variations in the proportions of the three major components of Chlorella as the R-value changes. This change is summarized in table IV for the highest, lowest

TABLE IV

CONSTITUENTS OF CHLORELLA CALCULATED FROM R-VALUE

R-VALUE	PROTEIN %	CARBOHYDRATE %	LIPID %
38	58.0	37.5	4.5
42	50.0	32.2	17.7
50	28.3	26.2	45.5
56	15.7	19.0	65.3
63	8.7	5.7	85.6

and intermediate R-values. These figures indicate that there is nearly a linear relation between the increases in R-value and per cent. lipid. The decreases in per cent. carbohydrate and per cent. protein are roughly linear as the R-value increases.

This means of calculating the composition of Chlorella from elementary analysis and R-values has proved to be trustworthy. The calculated percentages of lipid agreed closely with the values which were obtained by solvent extraction. The analyses of Chlorella of different R-values, made by means of solvent extraction, together with an analysis of the fatty acids of Chlorella, will be presented in another paper.

The Effect of Various Environmental Factors

A very considerable amount of work has been devoted to the study of methods of growing Chlorella and similar organisms to be used in investigations of photosynthesis. Valuable contributions to this end have been made through the publications of GAFFRON (**6**), SARGENT (**16**) EMERSON and LEWIS (**3**), PRATT (**11, 12, 13, 14**), TRELEASE and SELSAM (**19**), JACK MYERS (**7, 8, 9, 10**) and others. These investigations have done much to provide what is often regarded as a standard type of organism for photosynthetic studies, though, as MYERS (**7**) points out, "the photosynthetic mechanism has been given an appearance of stability which may not be warranted." The current concepts of the photosynthetic reactions are of necessity derived from observations made under relatively restricted en-

vironmental conditions, during short periods of time, and on the basis of certain assumptions concerning the nature of the products which are formed. We are here concerned less with the photosynthetic phenomena *per se* than with the products which the Chlorella cells are capable of producing under a variety of conditions. That the chemical composition of the cells varies greatly according to the conditions under which they are grown, becomes clearly evident. It need hardly be emphasized, however, that experiments of this type give little direct information as to whether the great variations in composition of the cells reflect differences in the course of the photosynthetic reactions.

In the study of a process as complex as the growth of a Chlorella culture, it is next to impossible to evaluate the effect of one environmental factor operating independently of others. Under the headings which follow, an attempt is made to evaluate the response of Chlorella due principally to variations imposed upon one environmental factor at a time. Generally, the response to variations of a single factor, the effect of which is being studied, depends upon the magnitude of the other factors which are operative.

An important consideration in this type of experimentation is the reproducibility of the results. All cultures were grown in sets of two or more, one culture in each set serving as the control while testing upon the others the effect of an environmental change. Duplicate cultures showed less than 5% difference in yield and less than 1% difference in R-value. Cultures grown under the same set of environmental conditions, but at different dates, showed nearly as good reproducibility. It was also found that a culture with any previously obtained yield and R-value could be grown again at will.

The great magnitude of the observed differences in composition of Chlorella grown under different environmental conditions raises the question, whether such differences in composition are entirely responses to environmental changes or, whether there is involved a selection of a strain of the organism which is adapted to growth under the special conditions. If a particular strain of Chlorella grew selectively under conditions producing high R-values, inoculation of new cultures with these cells of high R-value should give rise to more cells of high R-value. When this was done, the second culture, grown under the same conditions, did not reach as high R-value as the first. A third culture, inoculated with cells from the second, yielded cells of the usual low R-value, even though the three successive cultures all grew under conditions which brought about the production of high R-value cells from the usual inoculum. Also, cells from cultures of high R-value were used to inoculate new cultures which were then grown under conditions favoring the production of low R-value. These cultures yielded cells of low R-value. Apparently, then, the R-value attained by Chlorella depends upon environmental conditions during its growth rather than upon selective growth of a different strain.

Effect of Atmospheric Composition

Although the Chlorella cultures were grown in liquid media, they depended for their source of carbon upon the gas which was bubbled continuously through them. Thus, the composition of the gas stream is an important environmental factor. In most of this work the gas stream was either air or nitrogen, both enriched with 5% CO_2.

Carbon Dioxide. Cultures which depended upon air alone for their source of carbon grew very slowly and produced but one tenth the weight of cells produced by cultures which received air containing 5% CO_2. Comparison of cultures grown with 3% and 5% CO_2 in nitrogen showed no significant difference in yield during ten days at low light intensity, while at high light intensity the yields were roughly proportional to the CO_2 concentration. With 10% CO_2 in nitrogen and various light intensities, the yields were 77 to 87% of those when 5% CO_2 in nitrogen was used, and the R-values of the cultures with 10% CO_2 were also lower. These results are summarized in table V.

TABLE V

Comparative effect of different carbon dioxide concentration on yield and R-value of chlorella

Gas Mixture	Light, watts	Days	Yield, gm/2L.	R-Value
Air	200	15	0.1470	40.44
5% CO_2 + Air	200	15	1.4740	51.44
3% CO_2 + N_2	100	10	0.6617	43.73
5% CO_2 + N_2	100	10	0.6279	43.78
3% CO_2 + N_2	300	12	1.0625	44.18
5% CO_2 + N_2	300	12	1.4408	49.27
5% CO_2 + N_2	100	11	0.9760	45.66
10% CO_2 + N_2	100	11	0.6870	44.39
5% CO_2 + N_2	300	10	1.2508	52.12
10% CO_2 + N_2	300	10	0.9600	46.28

Oxygen. The century-old suggestion of Koene that the primitive atmosphere of the earth contained no free oxygen and that this gas was primarily the result of photosynthetic activity of early forms of plant life, has received considerable attention from geologists and cosmologists (**2, 15, 17**), but very little from plant physiologists. It is impossible to enter here upon a full discussion of this subject. Yet certain aspects thereof are sufficiently germane at least, to warrant their mention. Reference is made more particularly to the formation of coal and petroleum. It has been rather difficult to understand chemically the transformation into coal and petroleum of the constituents of vegetable cells and tissue as we know them today. On the other hand, if under conditions of low oxygen con-

tent of the atmosphere, plants produced compounds of a higher degree of reduction than at present, this may conceivably throw a different light on the problem. In terms of the present investigation, therefore, it is a question whether the presence of oxygen in the cultures of Chlorella affects the R-value of the cells.

It should be mentioned at the outset that our results on the culture of Chlorella with an oxygen-free gas stream in some respects are rather at variance with those of some other investigators. WARBURG (**20**) reported that Chlorella cells are not able to survive in cultures exposed to low partial pressures of oxygen. He ascribed this to nitrite poisoning. EMERSON, STAUFFER and UMBREIT (**4**) also reported that the cells cannot grow when

TABLE VI

THE EFFECT OF THE PARTIAL PRESSURE OF OXYGEN ON THE YIELD AND R-VALUE OF CHLORELLA

GAS MIXTURE 5% CO_2 IN:	LIGHT, WATTS	DAYS	% O	YIELD GM./2L.	R-VALUE
Air	100	11	20	0.7098	45.32
Nitrogen	100	11	trace	0.6912	45.51
Air	200	15	20	1.4740	51.44
Nitrogen	200	15	trace	1.8980	55.21
Oxygen	100	17	95	0.5034	38.67
Oxygen	200	17	95	0.4155	37.92

deprived of oxygen. In contrast with these findings, we obtained excellent growth of cultures when these were supplied with gas mixtures containing only traces or no oxygen, both when fixed nitrogen was supplied in the form of ammonium salts and as potassium nitrate. WARBURG and NEGELEIN (**21**) also found that the rate of photosynthesis of Chlorella decreases when the oxygen pressure is increased from 1/50 to one atmosphere. The experiments here recorded, table VI, show that high concentrations of oxygen result in appreciable differences in both yield and R-value as compared with cultures grown in a gas mixture of carbon dioxide in air or in nitrogen.

A mixture of 5% CO_2 and 95% O_2 was used under different culture conditions. Negligible growth was found in a culture after seven days, daylight illumination, with this gas mixture. Cultures illuminated by 100-watt lamps, one continuously and one intermittently, for 35 days, produced less than half the yield of similar cultures which received 5% CO_2 in air or in nitrogen, and the R-values were lower. These three cultures were grown in a medium containing KNO_3. When the nitrogen was supplied as NH_4Cl, even more striking results were obtained by the use of 5% $CO_2 + O_2$. Two cultures, illuminated by 100-watt and 200-watt lamps

respectively, for 17 days, produced but one third the yield of Chlorella obtained with 5% CO_2 in air or in nitrogen, and the R-values were the lowest ones obtained, 38.67 and 37.92. It is noteworthy that the culture illuminated with a 200-watt lamp produced lower yield and R-value than the culture illuminated with a 100-watt lamp. This is exactly opposite to the effect of higher light intensity upon cultures grown in 5% CO_2 in air or in nitrogen.

A mixture of 5% CO_2 in air contains about 20% oxygen, and the 5% CO_2 in nitrogen which was used contained only a trace of oxygen. Cultures grown for less than two weeks at a low light intensity produced approximately the same yields of cells, of about the same R-value, when either gas mixture was used. Under higher light intensity, the yields were larger and the R-values one to three units higher in cultures grown with the nitrogen mixture than in those which were grown with the air mixture, other conditions being equal. The difference in yield and R-value attained in the two gas mixtures became larger with longer times of growth. This effect was noted both in a medium which produced Chlorella of high R-value and in one which produced large yields of cells of low R-value.

Under intermittent illumination at high intensity, cultures grown with 5% CO_2 in air produced higher yields and R-values than did cultures which received the same exposure to light applied continuously. This was in contrast to the effect noted in the use of 5% CO_2 in nitrogen, as will be discussed more fully in the section on illumination.

Three cultures receiving 5% CO_2 and 95% hydrogen did not differ significantly in yield or R-value from comparable cultures grown with 5% CO_2 in nitrogen.

The effect of using a completely oxygen-free gas stream was tried by passing the 5% CO_2 in nitrogen through a hot combustion tube filled with copper before the gas was bubbled through the culture. Cultures grown under both continuous and intermittent illumination at low light intensity did not differ significantly in yield or R-value from the control cultures which grew with the untreated 5% CO_2 in nitrogen. Under the conditions used, the removal of the trace of oxygen from the gas mixture did not affect the growth of the culture.

Mineral Nutrients

In the course of these experiments a number of variations in the mineral nutrient solution were used. The total salt concentration as well as the proportions of the several ions in solution were varied. The objective was to find solutions suitable for growing cells of high R-value.

Table VII shows the molar concentrations of salts in the six media which were used for most of the experimental cultures. The essential difference between these media is the form in which fixed nitrogen was supplied. Those with nitrate are designated A, ammonium chloride B, and

ammonium phosphate C. The concentration of fixed nitrogen was the same in all these media except in A–2.

Each of the media had a pH of 5.9 when prepared. During growth, the cultures in A media became alkaline, as much as pH 7.2, whereas those in B media became acid, reaching pH 3.5 in old cultures. The change in pH was very small in C media.

It was found that the yield and R-value attained by a Chlorella culture was not determined by the medium alone. In general, cultures grown

TABLE VII

MOLAR CONCENTRATION OF SALTS IN CULTURE MEDIA

	A	A–2	B	B–2	C	C–2
KNO_3	0.00225	0.0250				
NH_4Cl			0.00225	0.00225		
$(NH_4)_2HPO_4$					0.000825	0.000825
$(NH_4)_2HPO_4$					0.000715	0.000715
KCl				0.0300		0.0300
$MgSO_4$	0.0100	0.0200	0.0100	0.0100	0.0100	0.0100
KH_2PO_4	0.0100	0.0180	0.0100	0.0100	0.0100	0.0100
[Fe]	0.000005	0.000005	0.000005	0.000005	0.000005	0.000005

in A–2 medium produced large yields of Chlorella having a relatively low R-value. B and C media were approximately equal in effect upon yield and R-value of cultures grown for short or intermediate times. For a long time of growth at high light intensity the C media were superior to the B in producing larger yields and higher R-values.

WATER. The local tap water supply comes from wells, and has a fairly high mineral content. Before using this water for preparation of culture media, it was boiled vigorously for 20 minutes to drive off the free chlorine. After it had cooled, the considerable quantities of carbonates which had been precipitated were removed by filtration. Nutrient solutions were then prepared by dissolving the mineral salts in this water. Micro-nutrients were not added, as the yields of Chlorella indicated that the water was adequate for providing micro-elements.

A comparison was made between tap water (treated as described) and distilled water, redistilled in all-glass apparatus. The salts of C–2 medium were dissolved in each water and the two cultures grew for 15 days, illuminated continuously with 200-watt lamps. The culture in the medium prepared with doubly distilled water produced only one-sixth the yield and had an R-value seven units lower than the other culture. All cultures here reported were grown in media prepared with tap water, treated as described above.

FIXED NITROGEN. While the R-value of Chlorella can be varied experimentally by imposing a change in any one of several environmental conditions, the extent of the variation in R-value is limited primarily by one

factor. This key factor which determines whether a culture will reach a high R-value is the supply of fixed nitrogen in the medium.

The fixed nitrogen content of the medium for each culture was computed from the nitrogen content of the salts added. From the weight of Chlorella produced and the per cent. nitrogen in the cells, the total nitrogen content of each crop of Chlorella was calculated. The fixed nitrogen remaining in the medium at the time the cells were harvested was estimated as the difference between the original fixed nitrogen and the nitrogen content of the Chlorella cells. This quantity is termed residual fixed nitrogen.

When the residual fixed nitrogen is plotted against R-value, certain relations become clear. No culture reached an R-value higher than 47 when the residual fixed nitrogen concentration was greater than 0.001 molar. Below 0.001 M, the R-value appears to be controlled by environmental factors other than nitrogen concentration. A number of cultures grown under low light intensity and for relatively short time reached residual fixed nitrogen concentrations between 0.001 M and zero, without attaining R-values as high as 47. With higher light intensity and long enough time, all of our cultures in which the residual fixed nitrogen was less then 0.001 M reached high R-values. For R-values of 57 and higher, the residual fixed nitrogen concentrations were below 0.0004 M. With the highest favorable light intensities, and even very long periods of time, if the residual fixed nitrogen concentration was greater than 0.001 M, the R-value remained below 47. Therefore, success in growing Chlorella of high R-value depends primarily upon the fixed nitrogen concentration of the medium.

The fact that an exiguous supply of nitrogen nutrients results in increased fat production in diatoms was observed by BEIJERINCK (1). Although these observations were based only upon microscopic examination, they have been entirely confirmed, in principle, by the methods of analysis and with the organism we have used.

If the medium initially contains so much fixed nitrogen that Chlorella cells growing in it do not reduce the concentration below 0.001 M, a culture of high R-value will not be obtained. In these experiments, a high R-value was never found for Chlorella grown in a medium containing 0.025 M KNO_3. When a culture is started with a fixed nitrogen concentration of 0.001 M or less, cells of high R-value are obtained but the yields are extremely small. Larger yields of cells can be obtained by supplying an adequate quantity of fixed nitrogen. With growth of the cells, the fixed nitrogen concentration decreases below 0.001 M and high R-values are obtained. We empirically selected an initial nitrogen concentration of 0.00225 M as suitable for this purpose. Concentrations of ammonium nitrogen much larger than 0.00225 M appeared to have an unfavorable effect upon the growth of Chlorella. Probably, by adjustment of other factors, a somewhat higher concentration of nitrate nitrogen would still permit pro-

duction of cells of high R-value, but only after inconveniently long periods of growth.

Cultures grown in medium A, B and C, all with the same fixed nitrogen concentration, produce about the same yields and R-values under low intensity illumination and relatively short periods of growth. With high light intensity and longer time, the yield increases more rapidly in A medium and the R-value shows more rapid increase in the B and C medium. Finally, after a long time at high light intensity, cultures in the three media approach equality both in yield and in R-value. The greater length of time required for attainment of high R-value in KNO_3 medium makes it less desirable than media containing ammonium nitrogen for the production of Chlorella of high R-value.

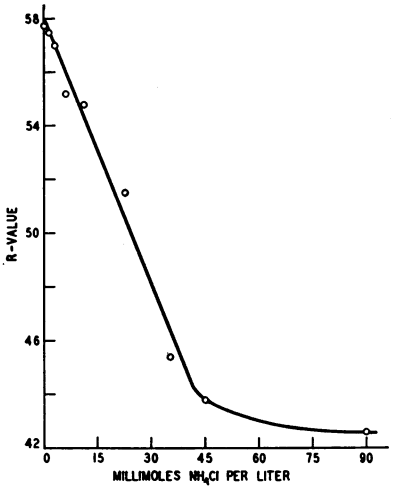

FIG. 1. Effect of nitrogen concentration in culture medium on the R-value of Chlorella.

In the presence of an abundant nitrogen supply, A–2 medium, Chlorella cells remained dark green for as long as we have maintained a culture, 112 days. Cell division ceases after about two weeks. After that time the cell count of a culture does not change, but the weight of the cells may increase by a factor of four to five. This increase in weight is accompanied by a slow increase in R-value from about 42 to about 47, and an increase in cell size which is readily apparent under the microscope.

Entirely different, both in visual appearance and chemical composition, is the course of development of a culture in a medium limited in fixed nitrogen. In the early stages of growth, the appearance, yield and R-value of the cells approximate those of cultures in A–2 medium. When cell division stops, either from age or depletion of the supply of fixed nitrogen, the R-value rises sharply and the appearance of the cells changes. The dark green color becomes lighter, gradually changing to yellow in the cultures of highest R-value. These pale Chlorella cells of high R-value were many

times the size of the dark green cells of low R. The pigment changes will be described in a following section.

The fixation of molecular nitrogen by green algae is a controversial subject. We have no critical evidence for or against nitrogen fixation, but have observed that in a few cultures of high R-value the nitrogen content of the cells was substantially more than the fixed nitrogen supplied to the cultures. This suggests the possibility that Chlorella may, under the special conditions producing high R-value, fix atmospheric nitrogen. Valid evidence in support of this view could be obtained only by much more rigorous control of conditions and refinement of analysis than was used in the experiments described here.

Figure 1 shows the effect of fixed nitrogen concentration in the medium upon the R-value of the Chlorella cells. Series of cultures in B medium

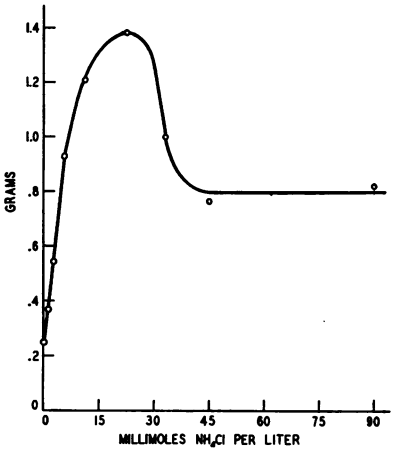

FIG. 2. Effect of nitrogen concentration in culture medium on the yield of Chlorella.

modified to contain varying initial nitrogen content were grown for 15 days under illumination with 200-watt lamps. The fixed nitrogen concentrations tested were 0 to 0.009 M. The yield in grams for the same cultures is shown in figure 2. The maximum yield was obtained with an initial fixed nitrogen concentration of about 0.00225 M. On either side of this concentration the yield decreased. As the nitrogen is reduced from 0.00337 M to almost zero, the R-value increases sharply. Later it was found that by increasing the time of growth of cultures as the nitrogen concentrations were increased from near zero to 0.00225, just as high R-values were obtained at any of these nitrogen concentrations, and that the yield was roughly proportional to the nitrogen concentration. This was not the case with nitrogen concentrations higher than 0.00225. Similar results were obtained from experiments in which different light intensities and different lengths of time were used.

When all the salts of the B medium were varied in the same proportion,

the effect on R-value of the cultures was not significantly different from that of varying the NH_4Cl alone. An increase in all of the salts was reflected in a small increase in yield.

MAGNESIUM. TRELEASE and SELSAM (19) have shown that although Chlorella exhibits a tolerance to high concentrations of magnesium, the yield of cells is small. In order to test the effect of lower concentrations of magnesium, a series of cultures was grown in C–2 medium and modifications in which the magnesium concentration was reduced to 0.005, 0.0025 and 0.001 M with the addition of corresponding quantities of Na_2SO_4 to maintain the same $[SO_4]$ in all. Only slightly higher yields with approximately the same R-value were produced in the three media with reduced magnesium. A similar series of cultures was grown in B–2 medium and modifications with 0.005, 0.0025 and 0.001 M magnesium, the $[SO_4]$ being maintained constant by the addition of K_2SO_4. In this series the yield and R-value decreased with decreasing magnesium. When $MgSO_4$ was omitted from the medium no discernable growth of Chlorella occurred.

PHOSPHATE. Several series of cultures were grown in modifications of the C-medium containing phosphate concentrations from 0.00125 to 0.04 M. The yield and R-values of these cultures did not differ significantly from those of the controls which contained 0.01 M phosphate. A culture to which no KH_2PO_4 was added produced only 2% of the yield of the control.

POTASSIUM. The reduction of [K] from 0.01 to 0.001 M was accompanied by a decrease of 10% in yield and of 2.5 units in R-value. The effect of increasing [K] was much more striking. Series of cultures were grown in both B and C medium to which KCl was added to give 2, 3, 4, 6 and 8 times the [K] in the controls. The yields and R-values showed progressive increases with 2, 3 and 4 times [K]. With 6 and 8 times [K], these values decreased slightly from those obtained with 4 times [K].

The yield and R-values obtained from C medium with added [K] were higher than those obtained from B medium with corresponding additions of [K]. The C medium with 4 times [K] is designated C–2. This medium yielded the highest R-values obtained.

SODIUM. When NaCl was substituted in equimolar quantities for additions of KCl as described under the section on potassium, similar effects on yield and R-value were observed. With large additions of NaCl to C medium, 0.13, 0.18 and 0.23 M, the yields and R-values decreased to approximately 70% of the maximum.

TOTAL SALT CONCENTRATION. It became apparent that the dilute media frequently recommended for the culture of algae are not suitable for growing the heaviest yields of Chlorella. Beijerinck's, Knop's and Molisch's solutions, for example, have total salt concentrations of about 0.02 M and often are used in diluted form. The media which yielded our best crops of Chlorella had total salt concentrations of 0.052 and 0.063 M (C–2 and A–2).

IRON. The quantity of this element present in the water supply and as impurity in the nutrient salts added was probably sufficient for the needs of Chlorella. No significant difference was observed between cultures without $FeCl_3$ added and those with additions up to 0.00001 M.

ILLUMINATION

INTENSITY. When the other environmental conditions were favorable, cultures grew well under light intensities ranging from that provided by a 25-watt lamp to full sunlight. With 25-, 40-, 60-, 75-, and 100-watt lamps the yields in any one medium were approximately proportional to the light intensity. Increase in light intensity above 100 watts caused relatively smaller increases in yield. Under continuous illumination the maximum yields in B, B–2, C and C–2 media were obtained with 200-watt lamps, in medium A–2 with 300-watt lamps. Higher intensity, from a 500-watt lamp, caused a small drop in yield from the maximum. Cultures in 5-gallon bottles, A–2 medium, grown under natural illumination gave the largest yields when exposed to direct sunlight. The effect of light intensity upon the yield and R-value of a culture can hardly be evaluated without consideration of the time factor. The effect of various times of growth at different light intensities is discussed later.

INTERMITTENT ILLUMINATION. Under this heading are included cultures which received illumination from Mazda lamps controlled by a time switch. In the latter, the lights were on for 12 hours and off for 12 hours alternately.

When the gas stream was 5% CO_2 in nitrogen, the yields of Chlorella were very nearly proportional to the total time of illumination. That is, an intermittently illuminated culture produced about half the yield of one illuminated continuously for the same number of days. Also, the yields and R-values were nearly equal for an intermittently illuminated culture and one which received the same total time of illumination continuously.

A different effect was found when 5% CO_2 in air was used. Intermittently illuminated cultures produced 8 – 25% higher yields of cells with R-values 2–3 units higher than did companion cultures which received the same amount of illumination continuously. In this experiment eight cultures were started in the same medium at the same time, supplied with 5% CO_3 in air and illuminated by 200-watt lamps. The continuously illuminated cultures were harvested after 35, 49, 63, and 77 days; the intermittently illuminated ones after 70, 98, 126 and 154 days.

To summarize the conditions producing cells of the highest R-value: C–2 medium was superior to the others tested, 5% CO_2 in nitrogen was the most favorable gas mixture, and 200-watt continuous illumination was the best of those tested.

Groups of experiments were arranged so that one of a pair of intermittently illuminated cultures was harvested at the end of a light period, while the companion culture was harvested at the end of a dark period, each of

the pair having been exposed to the same number of periods of illumination. In each case, the yield was a little less, and the R-value a little higher for the culture harvested at the end of a dark period. These results indicated that it might be possible to increase the R-value of Chlorella by allowing the culture to stand for a longer time in the dark. Some cultures were allowed to stand in the dark up to sixteen days. In none of these cultures was the increase in R-value much over one unit and there was also a considerable loss in weight.

Temperature

In attempting to determine the effect of temperature on the yield of Chlorella cells, the results become complicated by the difference of behavior of the organism in different media. Moreover, light intensity appears to influence the response to temperature of the cultures growing in the same medium. The temperatures employed for these tests ranged from 10 to 40° by 5° intervals.

Cultures in B–2 medium produced the best yields of Chlorella at 20°. There was no visible growth at 30° and low light intensity, but at high light intensity moderately good growth was observed at 30°. Growth was very slow at 10° with either light intensity. The R-value was lower at 10° than at 20°, but did not show more than 2 units variation at temperatures from 15 to 30°.

Chlorella grown in A–2 medium and under high light intensity, produced the greatest yields at 25°, and as good yields at 30° as at 20°. At very high light intensity some growth took place at 35° but none at 40°. While no growth took place in a culture maintained constantly at 40°, this temperature may be exceeded for short times without harmful effects. This was demonstrated in connection with cultures grown for the large scale production of Chlorella in 5-gallon bottles in a greenhouse exposed to direct sunlight. Occasionally the temperature within these cultures was observed to reach 43°. No harmful effect was apparent, judged by subsequent growth of the cultures and by comparison of their yields with similar cultures which had not become as warm. On the other hand, exposure to 40° for only an hour killed the cells of Chlorella cultures which were growing in B medium at low light intensity.

The effect of growing cultures at 15° and 20° with intermittent illumination was not significantly different from that observed at the same temperatures with continuous light.

Storage of energy

In considering the conditions favorable to the production of Chlorella cells of a given composition, an incomplete judgment will be reached if attention is paid to yield alone, or to R-value alone. Because each of these quantities is variable over a large range, both should be considered in the evaluation of the productivity of a culture grown under a given set of en-

vironmental factors. Since the R-value is proportional to the heat of combustion per gram of material, the product of R-value times the yield in grams expresses the total energy stored by a Chlorella culture. This energy unit, the product of R-value and grams yield, is abbreviated to RG.

Of interest from the physiological viewpoint is the extent to which a culture can make use of the light energy available to it. At the beginning of these experiments it was planned to make measurements of the photosynthesis and respiration of Chlorella grown under conditions leading to the production of cells of different R-values. Wartime changes in staff necessitated abandonment of this phase of the investigation. Lacking di-

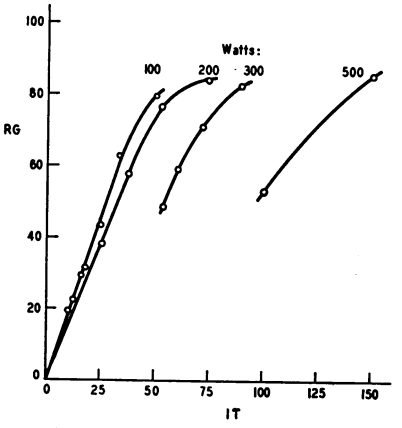

FIG. 3. Relation of RG to IT of Chlorella cultures in B medium under different light intensities.

rect physiological measurements, it becomes important to examine the available data for indications of possible differences in the utilization of light by cultures under various conditons.

We have no measure of the fracton of the incident light which was absorbed by the Chlorella cultures, although it was evident to the eye that this varied from near total absorption to almost complete transmission. We can, however, compare results obtained by growing Chlorella under different intensities of incident illumination. As we are concerned with the measurable over-all effect which has taken place during the entire period the culture was grown, a consideration of light intensity is meaningless without reference to the time factor. The manufacturer's rating of the initial lumens of the lamps served as the intensity factor, I. The time factor is expressed in days. The light energy incident upon a culture is proportional to the product of lumens times days. For convenience, this product is divided by 1000, and is denoted by the abbreviation IT.

When cultures are grown in the same medium and at the same light intensity, the value of RG is proportional to the value of IT over a considerable range. This is shown in figure 3. There is a deviation from the linear relationship as the cultures grow older, because the rate of increase

of both yield and R-value slows up. The curves lie quite close together when the intensity factor of IT is not greater than that which favors the greatest rate of growth of Chlorella in the given medium. For cultures in B medium, nearly the same RG is obtained at low values of IT with 25 to 200-watt lamps. Naturally, the same IT value will be reached in shorter time with higher light intensity. A 200-watt lamp is to be preferred for the attainment of maximum RG value of a culture growing in B medium. If the light intensity factor is greater than that which gives maximum growth, a larger value of IT is needed to produce a given value of RG, as shown by the curves for 300-watt and 500-watt lamps in figure 3.

The value of RG is not quite proportional to IT at very low values of the latter. While the weight factor of the quantity RG increases from the

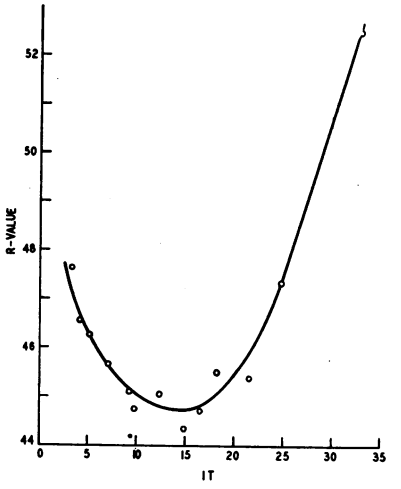

FIG. 4. R-value of Chlorella grown under low light intensity for relatively short times.

start with increase in IT, the R-value actually decreases at first, passes through a minimum at about IT = 15, then starts its steady increase at higher IT values. This is shown on a larger scale in figure 4. The points shown in this dip in the R-value curve are values from cultures grown with lamps of 100 watts and less for periods of 6 to 15 days. For example, an IT value of 15 is reached with a 100-watt lamp in 9.1 days, with 60-watt in 18 days, with 40-watt in 32 days, and with 25-watt in 55 days. With light intensities higher than that from a 100-watt lamp, the minimum R-value would probably be reached in a very much shorter time. Since high R-values were not attained with an IT value of less than 50, it is seen that it would require an inordinately long time to grow cultures of high R-value by use of lamps of small wattage.

The storage of energy as expressed by RG for a given value of IT is also influenced by the composition of the culture medium. This is illustrated by the curves in figure 5, showing the R-value, yield (grams) and RG for cul-

tures grown in media A–2 and C–2, each under the light intensity giving the best yields. This was 200 watts for C–2 and 300 watts for A–2. It is clear that the cells grown in C–2 medium exceed the others in both R-value and yield; hence the RG value is also higher. On the other hand, cells grown in A–2 medium, while producing relatively high yields, have a low R-value and consequently their RG is well below that of the cells grown in C–2 medium. In other words, when both are growing under the most favor-

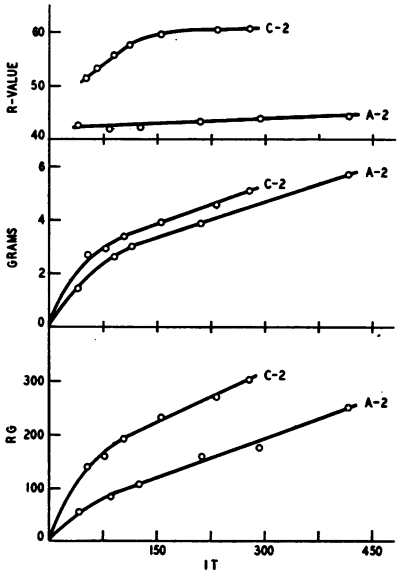

FIG. 5. Differences in the R-value, yield and RG of Chlorella cultures grown for long times at high light intensity in different media.

able conditions, the culture of high R-value stores considerably more energy than does the one of low R-value. Chlorella cells growing under conditions which produce high R-value appear to make more effective use of the incident light than cells grown under conditions producing low R-values.

One of the most striking effects noted in the use of different media was the unusually high yield of Chlorella obtained from A–2 medium at very low light intensity. In 15 days of continuous illumination with a 25-watt lamp, IT = 4, a culture in A–2 medium yielded 0.9 g. of cells. Under similar conditions, cultures in the other media yielded but 0.3 g.

The choice of a medium for Chlorella culture can be based upon two considerations, the chemical composition of cells which is desired, and the yield which is obtained for a given light exposure, the ratio of RG/IT. It is readily apparent that medium C–2 is definitely superior to A–2 for the production of large yields of Chlorella of high R-value. Medium A–2 is the best of the six if it is desired to grow the maximum amount of Chlorella at low light input, or if it is desired to obtain large quantities of cells having a low R-value.

In table VIII are shown high RG values attained by cultures in the various media. The highest RG values were obtained in media C–2 and A–2. It is probable that these values could have been increased if the experiments had been run for a longer time, that is, with higher IT.

Compared to the long continuing increase in RG with increase in IT obtained in C–2 and A–2 media, cultures in other media showed very little increase in RG, some after IT had reached 110, others after IT was only 55. Therefore, it was not profitable to grow cultures in the latter media

TABLE VIII

HIGHEST RG OBTAINED WITH DIFFERENT MEDIA.
(100 WATT, 1650 LUMENS; 200 WATT, 3700 LUMENS; 300 WATT, 5950 LUMENS.)

RG	R-VALUE	GM./2L.	IT	WATTS	DAYS	MEDIUM
305.6	59.56	5.1600	305.6	200	75	C–2
252.4	44.26	5.7020	416.5	300	70	A–2
192.4	56.43	3.4100	103.6	200	28	C–2
136.7	44.26	3.0876	111.0	200	30	A–2
126.5	63.33	1.9980	104.0	100	63	C–2, ¼ N
126.0	56.24	2.2408	111.0	200	30	A
106.0	55.24	1.9192	111.0	200	30	B–2
112.1	42.51	2.6376	55.5	200	15	A–2
99.1	55.32	1.7920	55.5	200	15	C–2
93.9	61.59	1.5242	46.2	100	28	C–2, ¼ N
87.5	51.09	1.7130	55.5	200	15	A
84.8	54.39	1.5594	55.5	200	15	B–2
83.0	53.76	1.5444	55.5	200	15	B
78.1	52.28	1.4939	55.5	200	15	C

beyond these IT values. For comparison with media in the lower IT groups, results obtained with media C–2 and A–2 are included. It is evident that at the lower IT levels also, the C–2 and A–2 media produce the highest RG values. In this table are included cultures in C–2 medium containing ¼ the specified quantity of fixed nitrogen, designated C–2, ¼ N.

PIGMENTS

The color change from dark green to yellow-green and finally to yellow, which accompanies the increase in R-value of Chlorella is a very striking phenomenon. Although this color change was not followed through from the dark green to the yellow cells, pigment analyses were made on selected cultures by Dr. H. H. Strain of this laboratory.

The chlorophyll and carotene content of Chlorella both decrease as the R-value of the cells increases. Among Chlorella cultures of low R-value the chlorophyll content may vary ten-fold, depending upon the culture medium, light intensity and other conditions. This variation, however, is small in comparison to the difference in chlorophyll content between cultures of low R-value and of high R-value. In the latter, the chlorophyll content was only about 1/500, and in extreme cases 1/2000 that of the 6% by weight of chlorophyll found in cells of low R-value.

What is more striking is the relationship between chlorophyll content and cell yield in cultures which have reached high R-values. As their R-value increases, there is a regular decrease in chlorophyll content to about 0.012%. From this point, there is comparatively little change in the chlorophyll content; yet the weight of the cells may increase three-fold, with a corresponding decrease in percentage of chlorophyll.

The decrease in carotene content is about one-tenth as great as the change in chlorophyll content. Obviously this results in a change of the ratio of chlorophyll to carotene.

It appears possible that, at high R-values, the increase in cell yield may be independent of the chlorophyll concentration. This possibility, and other problems relating to the pigment content and increase in organic matter of cells of high R-value, together with their photosynthetic and respiratory activities, should be given more thorough investigation.

Summary

A method is described for determining the degree of reduction of the total organic matter of plant material from its elementary chemical composition. The degree of reduction is designated the R-value; it is proportional to the heat of combustion and is an expression of the energy content of the material. From the elementary analysis and R-value, it is possible to calculate the approximate carbohydrate, protein and lipid content of the plant material. It is shown that these components in Chlorella vary widely with different environmental conditions under which the cells are grown. For example, the lipid content varied from 4.5 to 85.6%.

The influence of various environmental factors on the chemical composition of Chlorella is described. These factors include carbon dioxide concentration; aerobic and anaerobic atmospheres; mineral nutrients, more particularly fixed nitrogen; illumination and temperature. Conditions were found which favor the production of cells containing a large amount of lipid, that is, cells of high R-value. In general, cells having low R-value are produced when the fixed nitrogen in the medium is above 0.001 M; below this concentration, cells of higher R-value can be obtained. High light intensity also favors the production of cells of high R-value. The interrelationship of nitrogen supply and illumination is described. The energy stored by cultures under different environmental conditions is considered in relation to light intensity and time.

Very striking changes in chlorophyll content of Chlorella cells occur with increase in their R-value, indicating that cells with high lipid content carry on photosynthesis with a chlorophyll content 1/500 to 1/2000 that of cells of low lipid content.

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